African Journal of Microbiology Research Vol. 6(6) pp. 1171-1177, 16 February 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1348
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Detecting of *Verticillium dahliae* on anise seeds using a new seed health testing technique

Ghoneem K. M.[1]*, Al Sahli, A. A.[2] and Rashad Y. M.[2]

[1]Seed Pathology Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.
[2]Biology Department, Teachers College, King Saud University, Riaydh , Saudi Arabia

Accepted 12 December, 2011

Verticillium dahliae **is a fungal plant pathogen that attacks a wide range of plants including anise causing a wilt disease. The pathogen grows slowly on anise seeds using the standard moist blotter (SMB) or deep-freezing blotter (DFB) methods. In these methods saprophytes and fast growing fungi impair the detection of such slow growing fungi. An attempt was carried out to overcome this problem on anise seeds by applying alkaline seed-bed (ASB) technique for detecting slow growing seed-borne fungi. Data showed significant increase in the incidence levels of *V. dahliae* (3.4 and 4.73%) when alkaline KOH or NaOH technique was applied as compared to 0.2 and 0.7%, in SBM and DFB methods, respectively. Moreover, the ASB technique proved to be an effective option for early detecting the pathogen on anise seeds after 4 days of incubation in compared to other methods. The *in vitro* study indicated that the alkaline pH is the favored condition by the fungus. However, *V. dahliae* was able to grow in a wide range of pH (3.5 to 12.5) with a pH optimum of 8 at which the highest growth rate was recorded (0.96 g.L^{-1}/day). The maximum glucose coefficient was achieved at pH 8, while the highest glucose utilization was recorded at pH 12.5. These results suggest that ASB technique is recommended for the detection of the alkalophilic *V. dahliae*.**

Key words: Alkaline seed-bed, *Pimpinella anisum*, seed health, Slow growing fungi, *Verticillium* wilt.

INTRODUCTION

Verticillium dahliae Kleb. is a highly destructive phytopathogenic fungus that causes *Verticillium* wilt disease in a wide range of plants. Over 300 woody and herbaceous plant species are known to be susceptible to this pathogen including anise (*Pimpinella anisum* L.) (Klosterman et al., 2009). *Verticillium* wilt is a soil-borne fungal disease that causes billions of dollars losses in annual crops worldwide, especially in irrigated regions (Pegg and Brady, 2002). The fungus survives in soil up to eight years in the form of tiny black resting structures called *microsclerotia*. The fungus also survives in or on plant seeds in the form of mycelium or conidia (Huang et al., 2004).

*Corresponding author. E-mail: khalid_ghoneem@yahoo.com.

Standard moist blotter (SMB) and deep-freezing blotter (DFB) methods are two techniques recommended by the International Seed Testing Association for detecting the seed-borne fungi. Both methods develop saprophytes which seriously impair the detection of pathogenic fungi (ISTA, 1999). In practice, slow-growing fungi, including *Verticillium* species were reported to produce poor mycelial growth and do not show their imperfect stage on seeds. They have usually been overgrown by others and therefore often recorded in too low percentages (Neergaard, 1979). Elwakil and Ghoneem (2002) developed an efficient technique for detecting the slow growing fungi on seeds, which is the alkaline seed-bed (ASB) technique. The present work was planned to investigate the efficacy of alkaline seed-bed (ASB) technique in detecting the slow growing seed-borne *V. dahliae* in anise seeds and study the effects of pH

variation on the growth of *V. dahliae.*

MATERIALS AND METHODS

Samples

Fifty seed samples of local anise cultivar collected from growing fields in different governorates regions in Egypt (Alexandria, Cario, Assiut, Damietta, Gharbia and Dakhlia) during 2006 to 2010 were used in this study.

Seed health testing

A total number of 400 seeds from each sample were used and the percentages of fungi recovered using the following methods were tabulated.

Standard moist blotter (SMB)

Twenty-five seeds were planted in a 9 cm diameter Petri-dish containing three layers of blotter paper moistened with sterilized water. The plates were incubated at 20 ± 2°C for 7 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness.

Deep freezing blotter (DFB)

After planting seeds as described in the SMB method, the dishes were incubated at 20°C for 24 h and then transferred to a -20°C freezer for 24 h. This was followed by a 5-day incubation at 20 ± 2°C under cool white fluorescent lights with alternating cycles of 12 h light and 12 h darkness.

Alkaline seed-bed (ASB)

Three layers of blotter papers were soaked in KOH or NaOH solution at pH 10. The blotters were placed in 9-cm diameter Petri-dishes and 25 seeds were distributed on each seed-bed as described (ISTA, 1999). The plates were incubated at 20 ± 2°C under cool white fluorescent lights with alternating cycles of 12 h light and 12 h darkness. Pure cultures of the examined fungi were obtained using single spore and hyphal tip techniques. The isolated fungi were maintained on slants of potato carrot agar for further studies.

Identification of fungi

Fungal were identified according to their cultural properties, morphological and microscopical characteristics as described (Raper and Fennel, 1965; Ellis, 1971; Chidambaram et al., 1973; Domsch et al., 1980; Booth, 1977; Burgess et al., 1988; Moubasher, 1993). For determination of morphological structures, portions of fungal growth were mounted in lacto-phenol cotton blue stain on clean slides as proposed by Sime and Abbott (2002).

Effect of incubation period on recovery of *V. dahliae* using different methods

Three anise seed samples showing high incidence percentages of *V. dahliae* were selected according to the above seed health test. The anise seeds were tested using ASB method (KOH - NaOH) at the rate of 25 seeds per Petri dish in comparing with SMB and DFB methods. The pathogen incidence was recorded after 4, 7 and 10 days of incubation.

In vitro growth and glucose utilization of *V. dahliae* at different pHs

Verticillium dahliae was grown on plates of Czapek's Agar medium for 10 days at 25°C in darkness. The fungal growth was scraped gently from the medium surface by using a glass rod and suspended in sterile distilled water. The collected spores was adjusted to about 5 x 10^6 spores ml^{-1}, while 0.5 ml were used to inoculate 50 ml of Drews liquid medium (Drews, 1983) in 250 ml Erlenmeyer flasks. The growth media presented different pH values ranged from 3.5 to 12.2. The cultures were incubated at 20 ± 2°C for 7, 14 and 21 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness. The final culture pH of *V. dahliae* was measured at the end of incubation period. Cultures were then, filtered though filter paper (Whatman No.1) and dried at 80°C till constant weight. The fungal growth rate day^{-1} during the incubation periods was determined. Glucose residue in the cultural supernatant was estimated at the end of the incubation period using O-Toluidine method (Merck, 1974). The glucose coefficient was calculated as the following formula:

GC = Mycelial dry weight ($g.L^{-1}$) / Utilized glucose ($g.L^{-1}$)

Statistical analysis

Comparison of means was performed using Duncan's multiple range tests at $P \leq 0.05$ (Duncan, 1955) using the statistical analysis software "CoStat 6.4" (CoStat 2005).

RESULTS

Seed health testing

A total of 21 genera and 39 species of fungi were isolated from anise seeds using SMB, DFB and ASB techniques (Table 1). Of the three techniques, SMB yielded the highest number of fungi (21 genera and 37 species). Data revealed that *Alternaria alternata*, *Drechslera tertramera, Cladosporium* sp. and *Stemphlium* sp., were found to be the most common. Data indicated also that SBM technique enhanced the recovery of the fast growing saprophytes as *D. tetramera* (90%), *Stachybotrys atra* (82%), *Aspergillus niger* (74%) which commonly attack the non-viable seeds, while, DFB technique enhanced the recovery of *Cladosporium* sp., (92%) and *Stemphylium* sp., (98%).

In contrast, ASB technique increased the recovery of the slow growing fungi specially *Verticillium* species. *V. dahliae* (Figure 1) was the most frequently isolated species among *Verticillium* genus in KOH and NaOH treatments (62 and 60%, respectively) followed by *V. albo-atrum, V. chlamydosporium*, and *V. nubilum*. The incidence percentage of *V. dahliae* increased using ASB treatments (4.7 and 3.4 % in case of NaOH and KOH, respectively) comparing with SMB and DFB (0.2 and 0.7 %, respectively). ASB treatments increased also the

Table 1. Occurrence of anise seed-borne fungi using different seed health methods.

Fungus	SMB		DFB		ASB			
					KOH		NaOH	
	F %*	I %**	F %	I %	F %	I %	F %	I %
Alternaria alternata	100	21.0 b	98	31.24 a	94	18.98 b	98	26.16 ab
Alternaria radicina	50	3.48 b	42	2.88 b	44	4.07 b	54	6.22 a
Aspergillus flavus	68	4.96 a	20	0.7 b	28	1.30 b	26	1.13 b
A. nidulans	54	3.85 a	2	0.07 c	38	2.25 b	34	1.85 b
A. niger	74	3.50 a	6	0.16 b	12	0.50 b	24	0.7 b
A. ochraceus	10	0.53 bc	4	0.20 c	22	1.33 a	18	0.86 b
A. tamari	54	3.86 a	4	0.70 c	38	2.26 b	30	1.85 b
A. versicolor	28	0.86 b	24	1.13 b	40	1.82 a	30	1.39 ab
Cephalosporium acremonium	24	1.41 a	40	2.79 a	52	3.25 a	36	1.62 a
Chaetomium bostrychodes	20	1.16 a	2	0.16 b	0	0.00 b	0	0.00 b
Cladosporium sp.	72	11.11 b	92	19.93 a	84	14.9 ab	78	11.02 b
Curvularia lunata	16	1.00 a	8	0.36 b	8	0.60 b	10	1.00 a
Drechslera halodes	30	1.35 a	22	0.88 b	10	0.47 b	14	0.88 b
D. hawaiiensis	2	0.33 b	0	0.00 c	4	0.66 a	2	0.33 b
D. microba	4	0.40 b	0	0.00 c	6	0.60 b	2	1.00 bc
D. rostrata	6	0.60 a	4	0.40 ab	0	0.00 c	0	0.00 c
D. tetramera	90	5.04 a	62	2.04 b	50	1.85 b	58	2.02 b
Epicoccum purpurascens	54	2.84 a	44	3.15 a	28	1.11 b	36	1.23 b
Fusarium equiseti	20	0.84 b	18	0.68 b	30	0.68 b	30	1.68 a
F. oxysporum	14	0.75 b	14	0.91 ab	14	1.00 ab	16	1.16 a
F. incarnatum	20	0.94 bc	14	0.66 c	28	1.94 ab	32	2.22 a
F. solani	12	0.35 c	4	0.14 d	26	1.07 a	20	0.57 b
F. verticillioides	28	0.86 c	40	2.56 a	42	3.21 a	40	1.73 b
Humicola sp.	28	0.77 a	12	0.27 b	20	0.81 a	20	0.63 a
Mucor sp.	6	0.33 b	8	0.55 a	4	0.11 b	10	0.22 b
Myrothecium verrucaria	34	2.61 a	16	0.47 b	10	0.23 b	6	0.33 b
Nakataea sp.	28	1.33 a	14	0.44 b	6	0.16 bc	0	0.00 c
Nigrospora sp.	14	1.85 a	0	0.00 c	2	0.85 b	2	0.85 b
Penicillium sp.	62	3.45 a	50	2.05 b	44	1.62 b	42	1.72 b
Phoma sp.	6	0.33 a	8	0.16 a	2	0.16 a	4	0.33 a
Rhizopus stolonifer	68	3.11 a	8	3.08 a	2	0. 03 b	8	0.29 b
Stachybotrys atra	82	13.19 a	58	3.56 b	50	4.14 b	56	5.21 b
S. chartarum	20	6.50 a	19	2.02 b	16	2.50 b	17	2.30 b
Stemphylium sp.	90	9.02 b	98	12.42 a	88	6.62 b	100	9.00 b
Trichothecium sp.	6	0.27 b	14	0.81 a	4	0.18 bc	0	0.00 c
Verticillium albo-atrum	5	0.40 c	15	0.60 c	18	2.50 a	20	2.26 a
V. dahliae	10	0.20 c	24	0.70 c	62	3.40 b	60	4.73 a
V. chlamydosporium	0	0.00 c	2	0.33 b	6	1.33 a	6	1.50 a
V. nubilum	0	0.00 c	3	0.33 b	4	1.00 ab	5	1.33 a

* SMB = standard moist blotter, DFB = deep freezing blotter, ASB = alkaline seed-bed, F % = frequency percentage and I % = mean intensity percentage of infected seeds.
**Values of means within a row followed by the same letter(s) are not significantly different ($P \leq 0.05$)

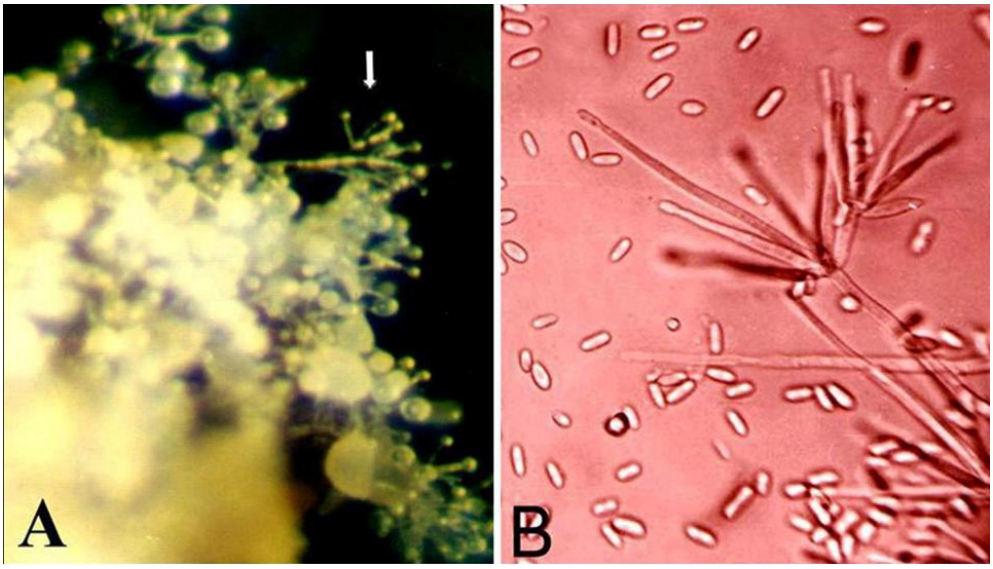

Figure 1. stereoscopic micrograph of *V. dahliae* on anise seed showing abundant white mycelium containing conidiophores bearing droplets of conidia (arrow) (A, X100). Microscopic photograph showing conidiophores and conidia of the fungus (B, X400).

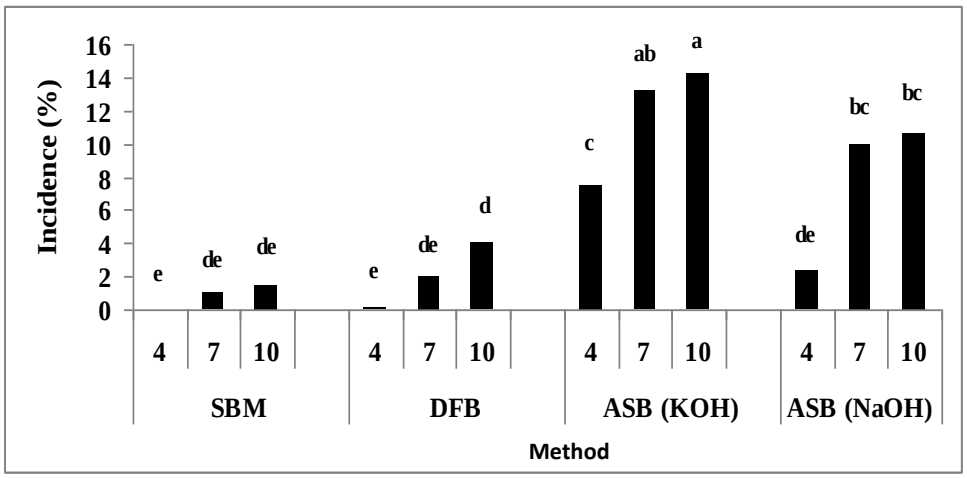

Figure 2. Effect of incubation period (4, 7 and 10 days) on the detection of *V. dahliae* on anise seeds, where, SMB = standard moist blotter, DFB = deep freezing blotter and ASB = alkaline seed-bed. - Columns superscripted with the same letter(s) are not significantly different at $P \leq 0.05$.

frequency of other fungi e.g. *Fusarium equiseti*, *F. incarnatum, F. solani* and *Cephalosporium acremonium* on anise seeds. On the other hand, the incidence of saprophytic fungi was lower in case of ASB than that of the other techniques. To the author's knowledge, this is the first report of the above mentioned seed-borne pathogens of anise in Egypt.

Effect of incubation period on the detection of *V. dahliae*

Incidence percentage of *V. dahliae* on anise seeds was investigated using the tested techniques after 4, 7 and 10 days of incubation (Figure 2). ASB technique detected *V. dahliae* faster and more efficiently than the other techniques. At day 4, the incidence percentages of *V. dahliae* in KOH and NaOH treatments were 7.5 and 2.3%, respectively as compared to 0.17% in DFB method, while, no incidence was recorded when SMB method was used. At day 7, the incidence percentages of *V. dahliae* in KOH and NaOH treatments were 13.7 and 9.9%, respectively comparing with 1.2% in SMB and 2.2% in DFB. At day 10, *V. dahliae* was detected in KOH and NaOH treatments at 14.2 and 10.7%, respectively

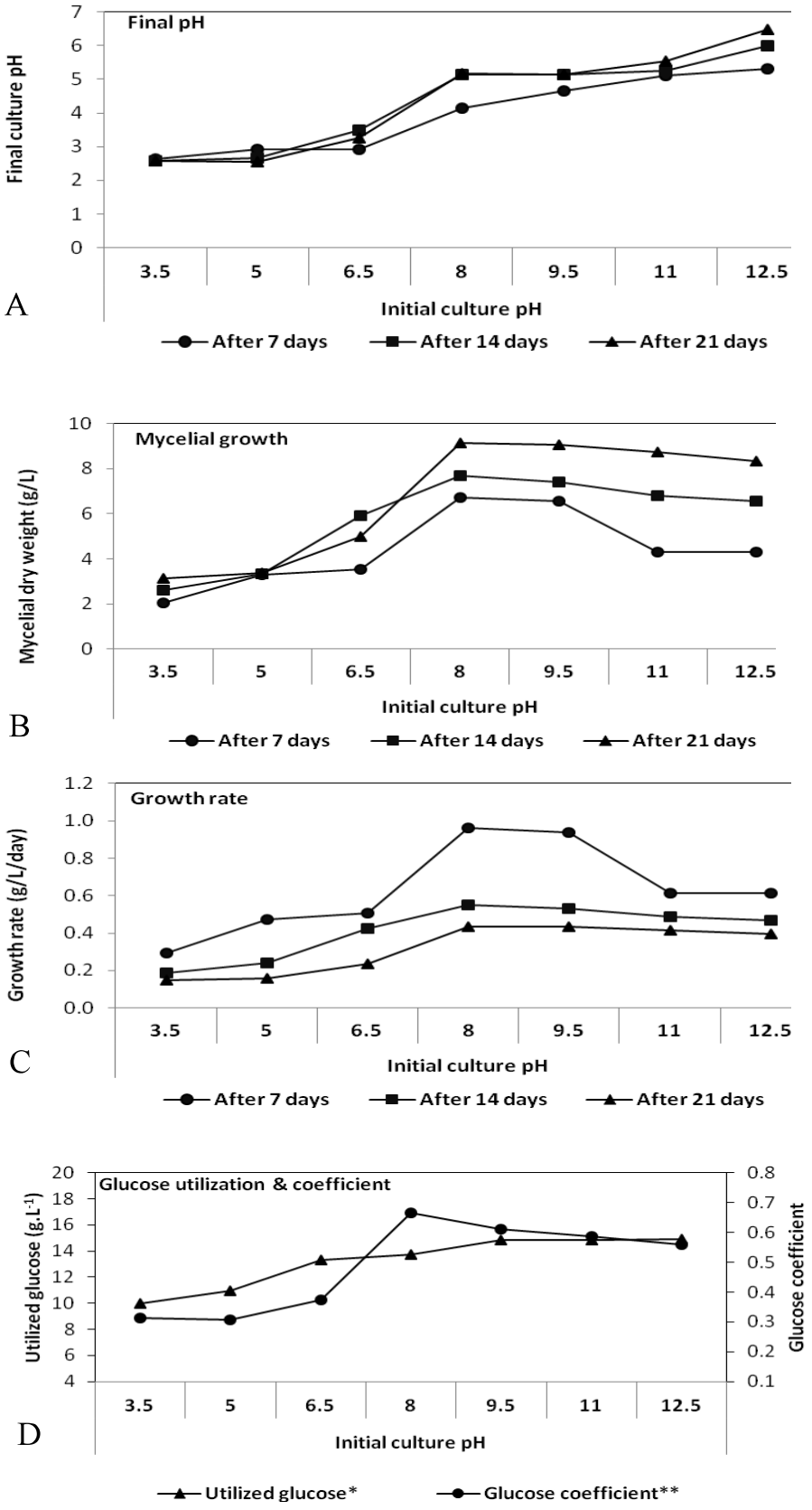

Figure 3. Effect of initial culture pH of V. *dahliae* on final culture pH, A: mycelial dry weight; B: growth rate; C: glucose utilization and coefficient; D: after 7, 14 and 21 days.

comparing with 1.7% in SMB and 4% in DFB.

Changes in the cultural pH

The relation between initial and final cultural pH was investigated after 7, 14 and 21 days of incubation. The fungus was allowed to grow at different initial pHs (3.5 to 12.5) and the final cultural pHs were recorded after 7, 14 and 21 days of incubation. Data illustrated in (Figure 3A) revealed that the increase in growth of *V. dahliae* leads to decrease in the final pH of the medium. However, the increase in initial pH of the medium led to a less acidic final pH. The differences between the initial and the final pHs were significantly decreased with the decrease in the initial pH. These results were observed after 7, 14 and 21 days.

Effect on mycelial growth of *V. dahliae*

Effect of initial pH on mycelial growth of *V. dahliae* after 7, 14 and 21 days of incubation is shown in Figure 3B. The results showed that, *V. dahliae* was able to grow in a wide range of pH (3.5 to 12.5). However, the fungal growth was significantly increased with the increase in the initial pH of the medium. The highest mycelial growth was recorded at pH 8 (9.14 $g.L^{-1}$) after 21 days of incubation after which the growth decreased. On the other hand, the growth was significantly increased with the increase in the incubation period.

Effect on the growth rate

The growth rate of *V. dahliae* was recorded at different inintial pH after 7, 14, 21 days of incubation (Figure 3C). Data revealed that the growth rate significantly increased with the increase in the initial pH. At day 7, the highest rate was recorded at pH 8 (0.96 $g.L^{-1}$/day). On the other hand, the growth rate decreased with the increase in incubation time.

Effect on glucose utilization and coefficient

Glucose utilization and coefficient of *V. dahliae* were studied at different pHs (3.5 to 12.5). Data illustrated in Figure 3D indicated that glucose utilization and coefficient significantly increased with the increase in the initial pH. The maximum glucose coefficient was achieved at pH 8, while the highest glucose utilization was recorded at pH 12.5.

DISCUSSION

Data of seed health testing revealed that ASB technique increased the recovery of *V. dahliae* comparing with SMB and DFB techniques. These results are in agreement with the findings of Elwakil and Ghoneem (2002) and Ghoneem et al. (2009) whom reported an abundant growth of *V. dahliae* when using ASB technique on fenugreek and fennel seeds, respectively. The efficacy of ASB technique in enhancement of *V. dahliae* recovery is attributed to the alkaline pH which is favored by the fungus. *V. dahliae* normally occurs in neutral to alkaline soils (pH 6 to 9), since acidic pH's (below 5.5) are inhibitory to pathogen growth and its microsclerotial production and survival (Pegg and Brady, 2002). Enhancement of the fungal growth by alkaline pH reflected on the incubation period needed for its recovery. The obtained results indicated that ASB technique detected *V. dahliae* faster and more efficiently than the other techniques (after 4 days). The success of ASB technique in early detection of the seed-borne *V. dahliae* and other slow growing fungi may be of a great importance in the agricultural quarantine tests of seeds.

In the present study, the results demonstrated that the increase in growth of *V. dahliae* led to a decrease in the final pH of the medium. This decrease in the pH can be attributed to the fungal metabolism which altered pH during their evolution. The results showed that, *V. dahliae* was able to grow in a wide range of pH (3.5 to 12.5). These findings are in agreement with that of Ghoneem et al. (2009) whom found that *V. dahliae* was able to grow in a wide range of pH with optimum pH 9. The ability of fungi to develop in a wide range of pH is partially due to adaptation associated with a genetic regulatory system that tailors gene expression to the ambient pH (Arst and Penalva, 2003). The present investigation revealed that the maximum growth rate and glucose coefficient of *V. dahliae* were recorded at pH 8. In this connection Abo-Ellil and Geweely (1999) reported that high alkalinity caused enhancement of sugar uptake by the facultative alkolophile *Verticillium lareririum* associated with higher reducing sugar contents and decreased polysaccharide accumulation in mycelial mats. It could thus be concluded that the increase in both of glucose utilization and glucose coefficient at high pH's is due to increased activity of amylase system which has pH optimum at the alkaline side. On another hand, hydrogen ion concentration in a medium could affect the fungal growth either indirectly by its effect on the availability of nutrients or directly by action on the cell surfaces (Wheeler et al., 1991). In this connection Schuldiner and Fishkes (1978) reported that Na/H antiporter catalyses the inward movement of protons in exchange of Na and that the following gradients are established in alkalophilic cells: Na out > Na in > H out. Non-alkalphiles lack the antiporter and cannot maintain a relatively acidified cytoplasm and hence have lost the ability to grow at alkaline pH. Similar conclusions were also reached by Horikoshi and Akiba (1982) and Koyama (1989) whom reported that internal pH of alkalophilic bacteria remains

relatively constant over a wide range of external pH values. This indicates a mechanism for the return of the protons into the cell by using antiporter systems for the removal of cations from internal spaces, or by exchanging the cations for protons depending on the presence of Na through the operation of a Na/H antiporter.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to the Research Center of Teachers College, King Saud University for funding this work through project No RSP-TCR-20.

REFERENCES

Abo-Ellil HA, Geweely NS (1999). Comparative biochemical studies on *Penicillium albicans* (Alkalosensitive) and *Verticillium lateritium* (Facultative Alkalophile). Pak. J. Biol. Sci., 2: 290-295.

Arst Jr HN, Penalva MA (2003). pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends Genet., 19: 224-231.

Booth C (1977). The genus *Fusarium*. Commonwealth Mycological Institute, Kew. Surrey, England.

Burgess LW, Liddell CM, Summerell BA (1988). Laboratory manual for *Fusarium* research. Incorporating a Key and descriptions of common species found in Australasia (2nd ed.). *Fusarium* research Lab., Plant Pathol. Dept and Agric. Entomol., Univ. Sydney press.

Chidambaram P, Mathur SB, Neergaard P (1973). Identification of seed borne *Drechslera* species. Danish Government Institute of Seed Pathology for Developing Countries, Hellerup, Copenhagen, Denmark Særtyk af FRIESIA X, 3: 165-207.

CoStat (2005). Cohort Software, 798 Lighthouse Ave. PMB 320 Monterey, USA.

Domsch KW, Gams W, Anderson TH (1980). Compendium of soil fungi. Academic Press, London, 1: 589.

Drews G (1983). Mikrobiologisches Praktikum. Springer Verlag Berlin, Germany.

Duncan DB (1955). Multiple range and multiple F test. Biometrics, 11: 1-42.

Ellis MB (1971). Dematiaceous Hyphomycetes. CMI, Kew, Surrey, England, p. 608.

Elwakil MA, Ghoneem KM (2002). An improved method of seed health testing for detecting the lurked seed-borne fungi of fenugreek. Pak. J. Plant Pathol., 1: 11-13.

Ghoneem KM, Saber WIA, Elwakil MA (2009). Alkaline seed-bed: an innovative technique for manifesting *Verticillium dahliae* on fennel seeds. Plant Pathol. J., 8(1): 22-26.

Horikoshi K, Akiba T (1982). Alkalophilic microorganisms, A new microbial world. Japan Scientific Societies Press, Tokyo and Springer Verlag, Berlin, p. 215.

Huang BL, Zhu H, Zhu F (2004). Affecting factors of the occurrence of *Verticillium* wilt of eggplant and the growth of *V. dahliae*. Phytophyl. Sinica, 31: 157-160.

ISTA (International Seed Testing Association) (1999). International Rules for Seed Testing. Rules 1999. Seed Sci. Technol., 24: 1-335.

Koyama N (1989). Na+-independence of the stability under alkaline conditions of the membrane of an alkalophilic *Bacillus***.** FEMS Microbiol. Lett., 61: 7-10.

Klosterman SJ, Atallah ZK, Vallad GE, Subbarao KV (2009) Diversity, pathogenicity and management of *Verticillium* species. Ann. Rev. Phytopathol., 47: 39–62.

Merck E (1974). Klinisches Labor 12 th ed. Merck, Darmstadt, p. 437.

Moubasher AH (1993). Soil fungi in Qatar and other Arab countries. The Center for Scientific and Applied Res. Univ. Qatar, Doha, Qatar**,** p. 566.

Neergaard P (1979). Seed Pathology. The MacMillan Press Ltd., London. Vols. 1 and 2.

Pegg GF, Brady BL (2002). *Verticillium* Wilts. CABI Publishing,Wallingford, UK.

Raper KE, Fennel DI (1965). The genus *Aspergillus.* The Williams and Wilkins Co., Baltimore, Maryland, p. 686.

Schuldiner S, Fishkes H (1978). Sodium-proton antiport in isolated membrane vesicles of *Escherichia coli*. Biochem., 17(4): 706–711.

Sime AD, Abbott SP (2002). Mounting medium for use in indoor air quality spore trap analyses. Mycol., 94: 1087-1088.

Wheeler KA, Hurdman BF, Pitt JI (1991). Influence of pH on the growth of some toxigenic species of *Aspergillus*, *Penicillium* and *Fusarium*. Int. J. Food Microbiol., 12: 141-150.